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(54) Title: NUCLEIC ACID BINDING PROTEINS			
(57) Abstract			
<p>The invention provides a method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a target nucleic acid sequence, wherein binding to the 5' base of the triplet by an <math>\alpha</math>-helical zinc finger nucleic acid binding motif in the protein is determined as follows: if the 5' base in the triplet is A, then position +6 in the <math>\alpha</math>-helix is Glu, Asn or Val; if the 5' base in the triplet is C, then position +6 in the <math>\alpha</math>-helix is Ser, Thr, Val, Ala, Glu or Asn.</p>			

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### Nucleic Acid Binding Proteins

The present invention relates to nucleic acid binding proteins. In particular, the invention relates to a method for designing a protein which is capable of binding to any predefined  
5 nucleic acid sequence.

Protein-nucleic acid recognition is a commonplace phenomenon which is central to a large number of biomolecular control mechanisms which regulate the functioning of eukaryotic and prokaryotic cells. For instance, protein-DNA interactions form the basis of the  
10 regulation of gene expression and are thus one of the subjects most widely studied by molecular biologists.

A wealth of biochemical and structural information explains the details of protein-DNA recognition in numerous instances, to the extent that general principles of recognition have  
15 emerged. Many DNA-binding proteins contain independently folded domains for the recognition of DNA, and these domains in turn belong to a large number of structural families, such as the leucine zipper, the "helix-turn-helix" and zinc finger families.

Despite the great variety of structural domains, the specificity of the interactions observed  
20 to date between protein and DNA most often derives from the complementarity of the surfaces of a protein  $\alpha$ -helix and the major groove of DNA [Klug, (1993) Gene 135:83-92]. In light of the recurring physical interaction of  $\alpha$ -helix and major groove, the tantalising possibility arises that the contacts between particular amino acids and DNA bases could be described by a simple set of rules; in effect a stereochemical recognition code which relates  
25 protein primary structure to binding-site sequence preference.

It is clear, however, that no code will be found which can describe DNA recognition by all DNA-binding proteins. The structures of numerous complexes show significant differences in the way that the recognition  $\alpha$ -helices of DNA-binding proteins from different structural  
30 families interact with the major groove of DNA, thus precluding similarities in patterns of recognition. The majority of known DNA-binding motifs are not particularly versatile, and

any codes which might emerge would likely describe binding to a very few related DNA sequences.

Even within each family of DNA-binding proteins, moreover, it has hitherto appeared that the deciphering of a code would be elusive. Due to the complexity of the protein-DNA interaction, there does not appear to be a simple "alphabetic" equivalence between the primary structures of protein and nucleic acid which specifies a direct amino acid to base relationship.

10 International patent application WO 96/06166 addresses this issue and provides a "syllabic" code which explains protein-DNA interactions for zinc finger nucleic acid binding proteins. A syllabic code is a code which relies on more than one feature of the binding protein to specify binding to a particular base, the features being combinable in the forms of "syllables", or complex instructions, to define each specific contact.

15 However, this code is incomplete, providing no specific instructions permitting the specific selection of nucleotides other than G in the 5' position of each triplet. The method relies on partial randomisation and subsequent selection in order to generate nucleic acid binding proteins for other specificities. Even with the aid of partial randomisation and selection, 20 however, neither the method reported in WO 96/06166 nor any other methods of the prior art have succeeded in isolating a zinc finger polypeptide based on the first finger of Zif268 capable of binding triplets wherein the 5' base is other than G or T. This is a serious shortfall in any ability to design zinc finger proteins.

## 25 Summary of the Invention

The present invention provides a more complete code which permits the selection of any nucleic acid sequence as the target sequence, and the design of a specific nucleic acid-binding protein which will bind thereto. Moreover, the invention provides a method by 30 which a zinc finger protein specific for any given nucleic acid sequence may be designed and optimised. The present invention therefore concerns a recognition code which has been

elucidated for the interactions of classical zinc fingers with nucleic acid. In this case a pattern of rules, expressing a bias in the use of amino acid residues for specific base recognition by zinc finger proteins, is provided which covers binding to all nucleic acid sequences.

5

According to a first aspect of the present invention, therefore, we provide a method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a nucleic acid base triplet in a target nucleic acid sequence, wherein binding to the 5' base of the nucleic acid base triplet by an  $\alpha$ -helical zinc finger nucleic acid binding motif in the protein is determined as follows:

10

- a) if the 5' base in the triplet is A, then position +6 in the  $\alpha$ -helix is Glu, Asn or Val;
- b) if the 5' base in the triplet is C, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val, Ala, Glu or Asn.

15

Preferably, binding to the 5' base of the triplet by an  $\alpha$ -helical zinc finger nucleic acid binding motif in the protein is additionally determined as follows:

20

- a) if the 5' base in the triplet is G, then position +6 in the  $\alpha$ -helix is Arg or Lys;
- b) if the 5' base in the triplet is T, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val or Lys.

25

All of the nucleic acid-binding residue positions of zinc fingers, as referred to herein, are numbered from the first residue in the  $\alpha$ -helix of the finger, ranging from +1 to +9. "-1" refers to the residue in the framework structure immediately preceding the  $\alpha$ -helix in a Cys2-His2 zinc finger polypeptide. Residues referred to as "++" are residues present in an adjacent (C-terminal) finger. Where there is no C-terminal adjacent finger, "++" interactions do not operate.

30

Cys2-His2 zinc finger binding proteins, as is well known in the art, bind to target nucleic acid sequences via  $\alpha$ -helical zinc metal atom co-ordinated binding motifs known as zinc fingers. Each zinc finger in a zinc finger nucleic acid binding protein is responsible for

determining binding to a nucleic acid triplet in a nucleic acid binding sequence. Preferably, there are 2 or more zinc fingers, for example 2, 3, 4, 5 or 6 zinc fingers, in each binding protein. Advantageously, there are 3 zinc fingers in each zinc finger binding protein.

- 5 The method of the present invention allows the production of what are essentially artificial nucleic acid binding proteins. In these proteins, artificial analogues of amino acids may be used, to impart the proteins with desired properties or for other reasons. Thus, the term "amino acid", particularly in the context where "any amino acid" is referred to, means any sort of natural or artificial amino acid or amino acid analogue that may be employed in
- 10 protein construction according to methods known in the art. Moreover, any specific amino acid referred to herein may be replaced by a functional analogue thereof, particularly an artificial functional analogue. The nomenclature used herein therefore specifically comprises within its scope functional analogues of the defined amino acids.
- 15 The  $\alpha$ -helix of a zinc finger binding protein aligns antiparallel to the nucleic acid strand, such that the primary nucleic acid sequence is arranged 3' to 5' in order to correspond with the N terminal to C-terminal sequence of the zinc finger. Since nucleic acid sequences are conventionally written 5' to 3', and amino acid sequences N-terminus to C-terminus, the result is that when a nucleic acid sequence and a zinc finger protein are aligned according
- 20 to convention, the primary interaction of the zinc finger is with the - strand of the nucleic acid, since it is this strand which is aligned 3' to 5'. These conventions are followed in the nomenclature used herein. It should be noted, however, that in nature certain fingers, such as finger 4 of the protein GLI, bind to the + strand of nucleic acid: see Suzuki *et al.*, (1994) NAR 22:3397-3405 and Paretich and Pabo, (1993) Science 261:1701-1707. The
- 25 incorporation of such fingers into nucleic acid binding molecules according to the invention is envisaged.

The invention provides a solution to a problem hitherto unaddressed in the art, by permitting the rational design of polypeptides which will bind nucleic acid triplets whose 5' residue is other than G. In particular, the invention provides for the first time a solution

30 for the design of polypeptides for binding triplets containing 5' A or C.

Preferably, if the 5' base in the triplet is C, then position +6 in the  $\alpha$ -helix is Ser, or Thr.

#### Brief Description of the Figures

5

Figure 1 illustrates zinc finger-DNA interactions. A: model of modular interactions with DNA base triplets in Zif268; B: similar model showing potential for synergism between zinc finger binding; C: model of library design for recognition code determination.

- 10 Figure 2 shows the amino acid sequence of three fingers used for phage display selection in the determination of recognition code.

Figure 3 lists the sequence-specific zinc finger clones obtained from phage selections, and their binding site signatures.

15

Figure 4 shows the base/amino acid correlation of the clones isolated from phage selections. Recognition patterns are highlighted.

- 20 Figure 5 illustrates the design of a zinc finger binding protein specific for a G12V mutant ras oncogene;

Figure 6 illustrates the binding specificity of the binding protein for the oncogene as opposed to the wild-type ras sequence; and

- 25 Figure 7 illustrates the results of an ELISA assay performed using the anti-ras binding protein with both wild-type and mutant target nucleic acid sequences.

### Detailed Description of the Invention

Position +6 in the  $\alpha$ -helix is generally responsible for the interaction with the 5' residue of a given triplet in the target. According to the present invention, a 5' A residue is preferentially bound by one of amino acids Val, Asn or Glu at position +6, while a 5' C residue is preferentially bound by Ala, Glu, Asn, Ser, Thr or Val, especially Ser or Thr.

The present invention concerns a method for preparing nucleic acid binding proteins which are capable of binding nucleic acid. Thus, whilst the solutions provided by the invention will result in a functional nucleic acid binding molecule, it is possible that naturally-occurring zinc finger nucleic acid binding molecules may not follow some or all of the rules provided herein. This does not matter, because the aim of the invention is to permit the design of the nucleic acid binding molecules on the basis of nucleic acid sequence, and not the converse. This is why the rules, in certain instances, provide for a number of possibilities for any given residue. In other instances, alternative residues to those given may be possible. The present invention, thus, does not seek to provide every solution for the design of a binding protein for a given target nucleic acid. It does, however, provide for the first time a complete solution allowing a functional nucleic acid binding protein to be constructed for any given nucleic acid triplet.

In a preferred aspect, therefore, the invention provides a method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a nucleic acid triplet in a target nucleic acid sequence, wherein binding to each base of the triplet by an  $\alpha$ -helical zinc finger nucleic acid binding motif in the protein is determined as follows:

- a) if the 5' base in the triplet is G, then position +6 in the  $\alpha$ -helix is Arg or Lys;
- b) if the 5' base in the triplet is A, then position +6 in the  $\alpha$ -helix is Glu, Asn or Val;
- c) if the 5' base in the triplet is T, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val or Lys;
- d) if the 5' base in the triplet is C, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val, Ala, Glu or Asn;
- e) if the central base in the triplet is G, then position +3 in the  $\alpha$ -helix is His;



- f) if the central base in the triplet is A, then position +3 in the  $\alpha$ -helix is Asn;  
g) if the central base in the triplet is T, then position +3 in the  $\alpha$ -helix is Ala, Ser or Val;  
provided that if it is Ala, then one of the residues at -1 or +6 is a small residue;  
h) if the central base in the triplet is C, then position +3 in the  $\alpha$ -helix is Ser, Asp, Glu,  
5 Leu, Thr or Val;  
i) if the 3' base in the triplet is G, then position -1 in the  $\alpha$ -helix is Arg;  
j) if the 3' base in the triplet is A, then position -1 in the  $\alpha$ -helix is Gln;  
k) if the 3' base in the triplet is T, then position -1 in the  $\alpha$ -helix is His or Thr;  
l) if the 3' base in the triplet is C, then position -1 in the  $\alpha$ -helix is Asp or His.

10

The foregoing represents a set of rules which permits the design of a zinc finger binding protein specific for any given nucleic acid sequence.

Expressed in an alternative form, the invention may be seen as an improvement in a method  
15 for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a nucleic acid triplet in a target nucleic acid sequence, said nucleic acid binding protein having at least one zinc finger nucleic acid binding motif, wherein binding to each base of the triplet by the zinc finger nucleic acid binding motif in the protein is determined as follows:

20

- a) if the 5' base in the triplet is G, then position +6 in the  $\alpha$ -helix is Arg or Lys;  
b) if the 5' base in the triplet is T, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val or Lys;  
c) if the central base in the triplet is G, then position +3 in the  $\alpha$ -helix is His;  
25 d) if the central base in the triplet is A, then position +3 in the  $\alpha$ -helix is Asn;  
e) if the central base in the triplet is T, then position +3 in the  $\alpha$ -helix is Ala, Ser or Val;  
provided that if it is Ala, then one of the residues at -1 or +6 is a small residue;  
f) if the central base in the triplet is C, then position +3 in the  $\alpha$ -helix is Ser, Asp, Glu,  
Leu, Thr or Val;  
30 g) if the 3' base in the triplet is G, then position -1 in the  $\alpha$ -helix is Arg;  
h) if the 3' base in the triplet is A, then position -1 in the  $\alpha$ -helix is Gln;

- i) if the 3' base in the triplet is T, then position -1 in the  $\alpha$ -helix is His or Thr;
- j) if the 3' base in the triplet is C, then position -1 in the  $\alpha$ -helix is Asp or His.

the improvement comprising determining the binding to the 5' of the triplet by the zinc  
5 finger nucleic acid binding motif in the protein as follows:

- k) if the 5' base in the triplet is A, then position +6 in the  $\alpha$ -helix is Glu, Asn or Val;
- l) if the 5' base in the triplet is C, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val, Ala, Glu or Asn.

10

Moreover, residue ++2 (residue +2 of the C-terminal adjacent zinc finger) influences binding to the 5' base of the triplet. Advantageously, therefore, a suitable ++2 residue is selected (see WO 96/06166).

- 15 Although zinc finger polypeptides are normally considered to bind to triplet sequences, the method of the present invention allows polypeptides to be designed to bind to target sequences which are not multiples of triplets. For example, a zinc finger polypeptide may be designed to bind to a palindromic target sequence. Such sequences are commonly found as, for example, restriction enzyme target sequences.

20

Preferably, creation of zinc fingers which bind to fewer than three nucleotides is achieved by specifying, in the zinc finger, amino acids which are unable to support H-bonding with the nucleic acid in the relevant position.

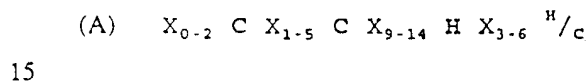
- 25 Advantageously, this is achieved by substituting Gly at position -1 (to eliminate a contact with a 3' base) and/or Ala at positions +3 and/or +6 (to eliminate contacts at the central or 5' base respectively).

- 30 Preferably, the contact with the final (3') base in the target sequence should be strengthened, if necessary, by substituting a residue at the relevant position which is capable of making a direct contact with the phosphate backbone of the nucleic acid.

A zinc finger binding motif is a structure well known to those in the art and defined in, for example, Miller *et al.*, (1985) EMBO J. 4:1609-1614; Berg (1988) PNAS (USA) 85:99-102; Lee *et al.*, (1989) Science 245:635-637; see International patent applications WO 96/06166 and WO 96/32475, corresponding to USSN 08/422,107, incorporated herein by reference.

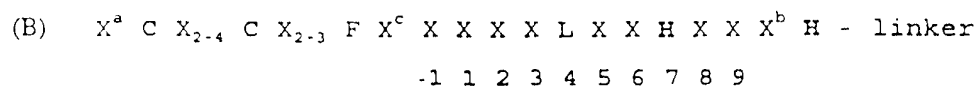
As used herein "nucleic acid" refers to both RNA and DNA, constructed from natural nucleic acid bases or synthetic bases, or mixtures thereof. Preferably, however, the binding proteins of the invention are DNA binding proteins.

In general, a preferred zinc finger framework has the structure:



where X is any amino acid, and the numbers in subscript indicate the possible numbers of residues represented by X.

In a preferred aspect of the present invention, zinc finger nucleic acid binding motifs may be represented as motifs having the following primary structure:



wherein X (including  $X^a$ ,  $X^b$  and  $X^c$ ) is any amino acid.  $X_{2-4}$  and  $X_{2-3}$  refer to the presence of 2 or 4, or 2 or 3, amino acids, respectively. The Cys and His residues, which together co-ordinate the zinc metal atom, are marked in bold text and are usually invariant, as is the Leu residue at position +4 in the  $\alpha$ -helix.

Modifications to this representation may occur or be effected without necessarily abolishing zinc finger function, by insertion, mutation or deletion of amino acids. For example it is

known that the second His residue may be replaced by Cys (Krizek *et al.*, (1991) J. Am. Chem. Soc. 113:4518-4523) and that Leu at +4 can in some circumstances be replaced with Arg. The Phe residue before  $X_c$  may be replaced by any aromatic other than Trp. Moreover, experiments have shown that departure from the preferred structure and residue assignments for the zinc finger are tolerated and may even prove beneficial in binding to certain nucleic acid sequences. Even taking this into account, however, the general structure involving an  $\alpha$ -helix co-ordinated by a zinc atom which contacts four Cys or His residues, does not alter. As used herein, structures (A) and (B) above are taken as an exemplary structure representing all zinc finger structures of the Cys2-His2 type.

Preferably,  $X^a$  is  $F/Y-X$  or  $P-F/Y-X$ . In this context, X is any amino acid. Preferably, in this context X is E, K, T or S. Less preferred but also envisaged are Q, V, A and P. The remaining amino acids remain possible.

Preferably,  $X_{2,4}$  consists of two amino acids rather than four. The first of these amino acids may be any amino acid, but S, E, K, T, P and R are preferred. Advantageously, it is P or R. The second of these amino acids is preferably E, although any amino acid may be used.

Preferably,  $X^b$  is T or I.

Preferably,  $X^c$  is S or T.

Preferably,  $X_{2,3}$  is G-K-A, G-K-C, G-K-S or G-K-G. However, departures from the preferred residues are possible, for example in the form of M-R-N or M-R.

Preferably, the linker is T-G-E-K or T-G-E-K-P.

As set out above, the major binding interactions occur with amino acids -1, +3 and +6.

Amino acids +4 and +7 are largely invariant. The remaining amino acids may be essentially any amino acids. Preferably, position +9 is occupied by Arg or Lys.

Advantageously, positions +1, +5 and +8 are not hydrophobic amino acids, that is to say are not Phe, Trp or Tyr. Preferably, position +2 is any amino acid, and preferably serine, save where its nature is dictated by its role as a +2 amino acid for an N-terminal zinc finger in the same nucleic acid binding molecule.

5

In a most preferred aspect, therefore, bringing together the above, the invention allows the definition of every residue in a zinc finger nucleic acid binding motif which will bind specifically to a given nucleic acid triplet.

- 10 The code provided by the present invention is not entirely rigid; certain choices are provided. For example, positions +1, +5 and +8 may have any amino acid allocation, whilst other positions may have certain options: for example, the present rules provide that, for binding to a central T residue, any one of Ala, Ser or Val may be used at +3. In its broadest sense, therefore, the present invention provides a very large number of proteins
- 15 which are capable of binding to every defined target nucleic acid triplet.

- Preferably, however, the number of possibilities may be significantly reduced. For example, the non-critical residues +1, +5 and +8 may be occupied by the residues Lys, Thr and Gln respectively as a default option. In the case of the other choices, for example,
- 20 the first-given option may be employed as a default. Thus, the code according to the present invention allows the design of a single, defined polypeptide (a "default" polypeptide) which will bind to its target triplet.

- In a further aspect of the present invention, there is provided a method for preparing a
- 25 nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a target nucleic acid sequence, comprising the steps of:

a) selecting a model zinc finger domain from the group consisting of naturally occurring zinc fingers and consensus zinc fingers; and

30

b) mutating at least one of positions -1, +3, +6 of the finger as required according to the rules set forth above.

In general, naturally occurring zinc fingers may be selected from those fingers for which the nucleic acid binding specificity is known. For example, these will be the fingers for which a crystal structure has been resolved: namely Zif 268 (Elrod-Erickson *et al.*, (1996) Structure 4:1171-1180), GLI (Pavletich and Pabo, (1993) Science 261:1701-1707), Tramtrack (Fairall *et al.*, (1993) Nature 366:483-487) and YY1 (Houbavij *et al.*, (1996) PNAS (USA) 93:13577-13582).

The naturally occurring zinc finger 2 in Zif 268 makes an excellent starting point from which to engineer a zinc finger and is preferred.

Consensus zinc finger structures may be prepared by comparing the sequences of known zinc fingers, irrespective of whether their binding domain is known. Preferably, the consensus structure is selected from the group consisting of the consensus structure P Y K C P E C G K S F S Q K S D L V K H Q R T H T G, and the consensus structure P Y K C S E C G K A F S Q K S N L T R H Q R I H T G E K P.

The consensuses are derived from the consensus provided by Krizek *et al.*, (1991) J. Am. Chem. Soc. 113:4518-4523 and from Jacobs, (1993) PhD thesis, University of Cambridge, UK. In both cases, the linker sequences described above for joining two zinc finger motifs together, namely TGEK or TGEKP can be formed on the ends of the consensus. Thus, a P may be removed where necessary, or, in the case of the consensus terminating T G, E K (P) can be added.

When the nucleic acid specificity of the model finger selected is known, the mutation of the finger in order to modify its specificity to bind to the target nucleic acid may be directed to residues known to affect binding to bases at which the natural and desired targets differ. Otherwise, mutation of the model fingers should be concentrated upon residues -1, +3, +6 and as provided for in the foregoing rules.

In order to produce a binding protein having improved binding, moreover, the rules provided by the present invention may be supplemented by physical or virtual modelling of the protein/nucleic acid interface in order to assist in residue selection.

5

Zinc finger binding motifs designed according to the invention may be combined into nucleic acid binding proteins having a multiplicity of zinc fingers. Preferably, the proteins have at least two zinc fingers. In nature, zinc finger binding proteins commonly have at least three zinc fingers, although two-zinc finger proteins such as Tramtrack are known.

- 10 The presence of at least three zinc fingers is preferred. Binding proteins may be constructed by joining the required fingers end to end, N-terminus to C-terminus. Preferably, this is effected by joining together the relevant nucleic acid coding sequences encoding the zinc fingers to produce a composite coding sequence encoding the entire binding protein. The invention therefore provides a method for producing a nucleic acid  
15 binding protein as defined above, wherein the nucleic acid binding protein is constructed by recombinant DNA technology, the method comprising the steps of:

- a) preparing a nucleic acid coding sequence encoding two or more zinc finger binding motifs as defined above, placed N-terminus to C-terminus;  
20 b) inserting the nucleic acid sequence into a suitable expression vector; and  
c) expressing the nucleic acid sequence in a host organism in order to obtain the nucleic acid binding protein.

- A "leader" peptide may be added to the N-terminal finger. Preferably, the leader peptide  
25 is MAEEKP.

- The nucleic acid encoding the nucleic acid binding protein according to the invention can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous nucleic acid into cells for  
30 either expression or replication thereof. Selection and use of such vehicles are well within the skill of the person of ordinary skill in the art. Many vectors are available, and selection

of appropriate vector will depend on the intended use of the vector, i.e. whether it is to be used for DNA amplification or for nucleic acid expression, the size of the DNA to be inserted into the vector, and the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and a signal sequence.

Both expression and cloning vectors generally contain nucleic acid sequence that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, polyoma, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors unless these are used in mammalian cells competent for high level DNA replication, such as COS cells.

Most expression vectors are shuttle vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA may also be replicated by insertion into the host genome. However, the recovery of genomic DNA encoding the nucleic acid binding protein is more complex than that of exogenously replicated vector because restriction enzyme digestion is required to excise nucleic acid binding protein DNA. DNA can be amplified by PCR and be directly transfected into the host cells without any replication component.



Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These can be obtained from *E. coli* plasmids, such as pBR322, Bluescript® vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both *E. coli* replication origin and *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up nucleic acid binding protein nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased, thereby leading to amplification (at its chromosomal integration site) of both the selection gene and the linked DNA that encodes the nucleic acid binding protein. Amplification is the process by which genes in greater demand for the production of a protein critical for growth, together with closely

associated genes which may encode a desired protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesised from thus amplified DNA.

- 5 Expression and cloning vectors usually contain a promoter that is recognised by the host organism and is operably linked to nucleic acid binding protein encoding nucleic acid. Such a promoter may be inducible or constitutive. The promoters are operably linked to DNA encoding the nucleic acid binding protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the  
10 vector. Both the native nucleic acid binding protein promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of nucleic acid binding protein encoding DNA.

Promoters suitable for use with prokaryotic hosts include, for example, the  $\beta$ -lactamase and  
15 lactose promoter systems, alkaline phosphatase, the tryptophan (Trp) promoter system and hybrid promoters such as the tac promoter. Their nucleotide sequences have been published, thereby enabling the skilled worker operably to ligate them to DNA encoding nucleic acid binding protein, using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno  
20 sequence operably linked to the DNA encoding the nucleic acid binding protein.

Preferred expression vectors are bacterial expression vectors which comprise a promoter of a bacteriophage such as phage  $\lambda$  or T7 which is capable of functioning in the bacteria. In one of the most widely used expression systems, the nucleic acid encoding the fusion  
25 protein may be transcribed from the vector by T7 RNA polymerase (Studier et al, Methods in Enzymol. 185; 60-89, 1990). In the *E. coli* BL21(DE3) host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the  $\lambda$ -lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG inducible lac UV5 promoter. This system has been employed successfully for over-production of many  
30 proteins. Alternatively the polymerase gene may be introduced on a lambda phage by infection with an int- phage such as the CE6 phage which is commercially available

(Novagen, Madison, USA). other vectors include vectors containing the lambda PL promoter such as PLEX (Invitrogen, NL) , vectors containing the trc promoters such as pTrcHisXpressTm (Invitrogen) or pTrc99 (Pharmacia Biotech, SE) or vectors containing the tac promoter such as pKK223-3 (Pharmacia Biotech) or PMAL (New England Biolabs, MA, USA).

Moreover, the nucleic acid binding protein gene according to the invention preferably includes a secretion sequence in order to facilitate secretion of the polypeptide from bacterial hosts, such that it will be produced as a soluble native peptide rather than in an inclusion body. The peptide may be recovered from the bacterial periplasmic space, or the culture medium, as appropriate.

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a *Saccharomyces cerevisiae* gene. Thus, the promoter of the TRP1 gene, the ADHI or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the  $\alpha$ - or  $\alpha$ -factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phosphoglycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, or a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PH05 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PH05 promoter is e.g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the PH05 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PH05 gene.

Nucleic acid binding protein gene transcription from vectors in mammalian hosts may be controlled by promoters derived from the genomes of viruses such as polyoma virus, adenovirus, fowlpox virus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus (CMV), a retrovirus and Simian Virus 40 (SV40), from heterologous mammalian  
5 promoters such as the actin promoter or a very strong promoter, e.g. a ribosomal protein promoter, and from the promoter normally associated with nucleic acid binding protein sequence, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding nucleic acid binding protein by higher eukaryotes may be  
10 increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent. Many enhancer sequences are known from mammalian genes (e.g. elastase and globin). However, typically one will employ an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270) and the CMV early promoter enhancer. The enhancer  
15 may be spliced into the vector at a position 5' or 3' to nucleic acid binding protein DNA, but is preferably located at a site 5' from the promoter.

Advantageously, a eukaryotic expression vector encoding a nucleic acid binding protein according to the invention may comprise a locus control region (LCR). LCRs are capable  
20 of directing high-level integration site independent expression of transgenes integrated into host cell chromatin, which is of importance especially where the nucleic acid binding protein gene is to be expressed in the context of a permanently-transfected eukaryotic cell line in which chromosomal integration of the vector has occurred, in vectors designed for gene therapy applications or in transgenic animals.

25 Eukaryotic vectors may also contain sequences necessary for the termination of transcription and for stabilising the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated  
30 portion of the mRNA encoding nucleic acid binding protein.

An expression vector includes any vector capable of expressing nucleic acid binding protein nucleic acids that are operatively linked with regulatory sequences, such as promoter regions, that are capable of expression of such DNAs. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector, that upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those with ordinary skill in the art and include those that are replicable in eukaryotic and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. For example, DNAs encoding nucleic acid binding protein may be inserted into a vector suitable for expression of cDNAs in mammalian cells, e.g. a CMV enhancer-based vector such as pEVRF (Matthias, et al., (1989) NAR 17, 6418).

Particularly useful for practising the present invention are expression vectors that provide for the transient expression of DNA encoding nucleic acid binding protein in mammalian cells. Transient expression usually involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector, and, in turn, synthesises high levels of nucleic acid binding protein. For the purposes of the present invention, transient expression systems are useful e.g. for identifying nucleic acid binding protein mutants, to identify potential phosphorylation sites, or to characterise functional domains of the protein.

Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing nucleic acid binding protein expression and function are known to those skilled in the art. Gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), or in situ hybridisation, using an appropriately labelled probe which may

be based on a sequence provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

In accordance with another embodiment of the present invention, there are provided cells  
5 containing the above-described nucleic acids. Such host cells such as prokaryote, yeast and higher eukaryote cells may be used for replicating DNA and producing the nucleic acid binding protein. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, such as *E. coli*, e.g. *E. coli* K-12 strains, DH5a and HB101, or Bacilli. Further hosts suitable for the nucleic acid binding protein encoding vectors include  
10 eukaryotic microbes such as filamentous fungi or yeast, e.g. *Saccharomyces cerevisiae*. Higher eukaryotic cells include insect and vertebrate cells, particularly mammalian cells including human cells, or nucleated cells from other multicellular organisms. In recent years propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are epithelial or fibroblastic cell  
15 lines such as Chinese hamster ovary (CHO) cells, NIH 3T3 cells, HeLa cells or 293T cells. The host cells referred to in this disclosure comprise cells in *in vitro* culture as well as cells that are within a host animal.

DNA may be stably incorporated into cells or may be transiently expressed using methods  
20 known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene, and growing the transfected cells under conditions selective for cells expressing the marker gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene to monitor transfection efficiency.

25 To produce such stably or transiently transfected cells, the cells should be transfected with a sufficient amount of the nucleic acid binding protein-encoding nucleic acid to form the nucleic acid binding protein. The precise amounts of DNA encoding the nucleic acid binding protein may be empirically determined and optimised for a particular cell and  
30 assay.

Host cells are transfected or, preferably, transformed with the above-captioned expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Heterologous DNA may be introduced into host cells by any method known in the art, such as transfection with a vector encoding a heterologous DNA by the calcium phosphate coprecipitation technique or by electroporation. Numerous methods of transfection are known to the skilled worker in the field. Successful transfection is generally recognised when any indication of the operation of this vector occurs in the host cell. Transformation is achieved using standard techniques appropriate to the particular host cells used.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g. Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press).

Transfected or transformed cells are cultured using media and culturing methods known in the art, preferably under conditions, whereby the nucleic acid binding protein encoded by the DNA is expressed. The composition of suitable media is known to those in the art, so that they can be readily prepared. Suitable culturing media are also commercially available.

In a further aspect, the invention also provides means by which the binding of the protein designed according to the rules can be improved by randomising the proteins and selecting for improved binding. In this aspect, the present invention represents an improvement of the method set forth in WO 96/06166. Thus, zinc finger molecules designed according to the invention may be subjected to limited randomisation and subsequent selection, such as by phage display, in order to optimise the binding characteristics of the molecule.

Preferably, therefore, the method according to the invention comprises the further steps of randomising the sequence of the zinc finger binding motifs at selected sites, screening the

randomised molecules obtained and selecting the molecules having the most advantageous properties. Generally, those molecules showing higher affinity and/or specificity of the target nucleic acid sequence are selected.

- 5 Mutagenesis and screening of target nucleic acid molecules may be achieved by any suitable means. Preferably, the mutagenesis is performed at the nucleic acid level, for example by synthesising novel genes encoding mutant proteins and expressing these to obtain a variety of different proteins. Alternatively, existing genes can be themselves mutated, such by site-directed or random mutagenesis, in order to obtain the desired mutant  
10 genes.

Mutations may be performed by any method known to those of skill in the art. Preferred, however, is site-directed mutagenesis of a nucleic acid sequence encoding the protein of interest. A number of methods for site-directed mutagenesis are known in the art, from  
15 methods employing single-stranded phage such as M13 to PCR-based techniques (see "PCR. Protocols: A guide to methods and applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (eds.). Academic Press, New York, 1990). Preferably, the commercially available Altered Site II Mutagenesis System (Promega) may be employed, according to the directions given by the manufacturer.

- 20 Screening of the proteins produced by mutant genes is preferably performed by expressing the genes and assaying the binding ability of the protein product. A simple and advantageously rapid method by which this may be accomplished is by phage display, in which the mutant polypeptides are expressed as fusion proteins with the coat proteins of  
25 filamentous bacteriophage, such as the minor coat protein pII of bacteriophage m13 or gene III of bacteriophage Fd, and displayed on the capsid of bacteriophage transformed with the mutant genes. The target nucleic acid sequence is used as a probe to bind directly to the protein on the phage surface and select the phage possessing advantageous mutants, by affinity purification. The phage are then amplified by passage through a bacterial host, and  
30 subjected to further rounds of selection and amplification in order to enrich the mutant pool for the desired phage and eventually isolate the preferred clone(s). Detailed methodology



for phage display is known in the art and set forth, for example, in US Patent 5,223,409; Choo and Klug, (1995) *Current Opinions in Biotechnology* 6:431-436; Smith, (1985) *Science* 228:1315-1317; and McCafferty *et al.*, (1990) *Nature* 348:552-554; all incorporated herein by reference. Vector systems and kits for phage display are available  
5 commercially, for example from Pharmacia.

Randomisation of the zinc finger binding motifs produced according to the invention is preferably directed to those residues where the code provided herein gives a choice of residues. For example, therefore, positions +1, +5 and +8 are advantageously  
10 randomised, whilst preferably avoiding hydrophobic amino acids; positions involved in binding to the nucleic acid, notably -1, +2, +3 and +6, may be randomised also, preferably within the choices provided by the rules of the present invention.

Preferably, therefore, the "default" protein produced according to the rules provided by the  
15 invention can be improved by subjecting the protein to one or more rounds of randomisation and selection within the specified parameters.

Advantageously, the zinc finger proteins according to the invention may be randomised such that 2 or more residues are randomised together. For example, it is preferred that  
20 residues -1 and +6 of adjacent zinc fingers in a zinc finger protein be randomised together. Preferably, position +6 of a zinc finger and positions -1, +1, +2 and +3 of an adjacent zinc finger are randomised together. This reflects cooperativity between adjacent zinc fingers and allows every possible triple junction base sequence to be specified.

25 Nucleic acid binding proteins according to the invention may be employed in a wide variety of applications, including diagnostics, therapy and as research tools. Advantageously, they may be employed as diagnostic tools for identifying the presence of nucleic acid molecules in a complex mixture. nucleic acid binding molecules according to the invention can differentiate single base pair changes in target nucleic acid molecules.

30

Accordingly, the invention provides a method for determining the presence of a target nucleic acid molecule, comprising the steps of:

- a) preparing a nucleic acid binding protein by the method set forth above which is specific  
5 for the target nucleic acid molecule; •
- b) exposing a test system comprising the target nucleic acid molecule to the nucleic acid binding protein under conditions which promote binding, and removing any nucleic acid binding protein which remains unbound;
- c) detecting the presence of the nucleic acid binding protein in the test system.

10 In a preferred embodiment, the nucleic acid binding molecules of the invention can be incorporated into an ELISA assay. For example, phage displaying the molecules of the invention can be used to detect the presence of the target nucleic acid, and visualised using enzyme-linked anti-phage antibodies.

15 Further improvements to the use of zinc finger phage for diagnosis can be made, for example, by co-expressing a marker protein fused to the minor coat protein (gVIII) of bacteriophage. Since detection with an anti-phage antibody would then be obsolete, the time and cost of each diagnosis would be further reduced. Depending on the requirements,  
20 suitable markers for display might include the fluorescent proteins (A. B. Cubitt, *et al.*, (1995) *Trends Biochem Sci.* 20, 448-455; T. T. Yang, *et al.*, (1996) *Gene* 173, 19-23), or an enzyme such as alkaline phosphatase which has been previously displayed on gIII (J. McCafferty, R. H. Jackson, D. J. Chiswell, (1991) *Protein Engineering* 4, 955-961).  
25 Labelling different types of diagnostic phage with distinct markers would allow multiplex screening of a single nucleic acid sample. Nevertheless, even in the absence of such refinements, the basic ELISA technique is reliable, fast, simple and particularly inexpensive. Moreover it requires no specialised apparatus, nor does it employ hazardous reagents such as radioactive isotopes, making it amenable to routine use in the clinic. The major advantage of the protocol is that it obviates the requirement for gel electrophoresis,  
30 and so opens the way to automated nucleic acid diagnosis.

The invention provides nucleic acid binding proteins which can be engineered with exquisite specificity. The invention lends itself, therefore, to the design of any zinc finger-comprising molecule of which specific nucleic acid binding is required. For example, the proteins according to the invention may be employed in the manufacture of chimeric  
5 restriction enzymes, in which a nucleic acid cleaving domain is fused to a nucleic acid binding domain comprising a zinc finger as described herein.

Moreover, the invention provides therapeutic agents and methods of therapy involving use of nucleic acid binding proteins as described herein. In particular, the invention provides  
10 the use of polypeptide fusions comprising an integrase, such as a viral integrase, and a nucleic acid binding protein according to the invention to target nucleic acid sequences *in vivo* (Bushman, (1994) PNAS (USA) 91:9233-9237). In gene therapy applications, the method may be applied to the delivery of functional genes into defective genes, or the delivery of nonsense nucleic acid in order to disrupt undesired nucleic acid. Alternatively,  
15 genes may be delivered to known, repetitive stretches of nucleic acid, such as centromeres, together with an activating sequence such as an LCR. This would represent a route to the safe and predictable incorporation of nucleic acid into the genome.

In conventional therapeutic applications, nucleic acid binding proteins according to the  
20 invention may be used to specifically knock out cell having mutant vital proteins. For example, if cells with mutant ras are targeted, they will be destroyed because ras is essential to cellular survival. Alternatively, the action of transcription factors may be modulated, preferably reduced, by administering to the cell agents which bind to the binding site specific for the transcription factor. For example, the activity of HIV tat may  
25 be reduced by binding proteins specific for HIV TAR.

Moreover, binding proteins according to the invention may be coupled to toxic molecules, such as nucleases, which are capable of causing irreversible nucleic acid damage and cell death. Such agents are capable of selectively destroying cells which comprise a mutation in  
30 their endogenous nucleic acid.

Nucleic acid binding proteins and derivatives thereof as set forth above may also be applied to the treatment of infections and the like in the form of organism-specific antibiotic or antiviral drugs. In such applications, the binding proteins may be coupled to a nuclease or other nuclear toxin and targeted specifically to the nucleic acids of microorganisms.

5

The invention likewise relates to pharmaceutical preparations which contain the compounds according to the invention or pharmaceutically acceptable salts thereof as active ingredients, and to processes for their preparation.

- 10 The pharmaceutical preparations according to the invention which contain the compound according to the invention or pharmaceutically acceptable salts thereof are those for enteral, such as oral, furthermore rectal, and parenteral administration to (a) warm-blooded animal(s), the pharmacological active ingredient being present on its own or together with a pharmaceutically acceptable carrier. The daily dose of the active ingredient depends on the
- 15 age and the individual condition and also on the manner of administration.

- The novel pharmaceutical preparations contain, for example, from about 10 % to about 80%, preferably from about 20 % to about 60 %, of the active ingredient. Pharmaceutical preparations according to the invention for enteral or parenteral administration are, for
- 20 example, those in unit dose forms, such as sugar-coated tablets, tablets, capsules or suppositories, and furthermore ampoules. These are prepared in a manner known per se, for example by means of conventional mixing, granulating, sugar-coating, dissolving or lyophilising processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active ingredient with solid carriers, if desired granulating a mixture
- 25 obtained, and processing the mixture or granules, if desired or necessary, after addition of suitable excipients to give tablets or sugar-coated tablet cores.

- Suitable carriers are, in particular, fillers, such as sugars, for example lactose, sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example
- 30 tricalcium phosphate or calcium hydrogen phosphate, furthermore binders, such as starch paste, using, for example, corn, wheat, rice or potato starch, gelatin, tragacanth,

methylcellulose and/or polyvinylpyrrolidone, if desired, disintegrants, such as the abovementioned starches, furthermore carboxymethyl starch, crosslinked polyvinylpyrrolidone, agar, alginic acid or a salt thereof, such as sodium alginate; auxiliaries are primarily glidants, flow-regulators and lubricants, for example silicic acid, talc, stearic acid or salts thereof, such as magnesium or calcium stearate, and/or polyethylene glycol. Sugar-coated tablet cores are provided with suitable coatings which, if desired, are resistant to gastric juice, using, inter alia, concentrated sugar solutions which, if desired, contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, coating solutions in suitable organic solvents or solvent mixtures or, for the preparation of gastric juice-resistant coatings, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate. Colorants or pigments, for example to identify or to indicate different doses of active ingredient, may be added to the tablets or sugar-coated tablet coatings.

Other orally utilisable pharmaceutical preparations are hard gelatin capsules, and also soft closed capsules made of gelatin and a plasticiser, such as glycerol or sorbitol. The hard gelatin capsules may contain the active ingredient in the form of granules, for example in a mixture with fillers, such as lactose, binders, such as starches, and/or lubricants, such as talc or magnesium stearate, and, if desired, stabilisers. In soft capsules, the active ingredient is preferably dissolved or suspended in suitable liquids, such as fatty oils, paraffin oil or liquid polyethylene glycols, it also being possible to add stabilisers.

Suitable rectally utilisable pharmaceutical preparations are, for example, suppositories, which consist of a combination of the active ingredient with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols or higher alkanols. Furthermore, gelatin rectal capsules which contain a combination of the active ingredient with a base substance may also be used. Suitable base substances are, for example, liquid triglycerides, polyethylene glycols or paraffin hydrocarbons.

Suitable preparations for parenteral administration are primarily aqueous solutions of an active ingredient in water-soluble form, for example a water-soluble salt, and furthermore suspensions of the active ingredient, such as appropriate oily injection suspensions, using suitable lipophilic solvents or vehicles, such as fatty oils, for example sesame oil, or  
5 synthetic fatty acid esters, for example ethyl oleate or triglycerides, or aqueous injection suspensions which contain viscosity-increasing substances, for example sodium carboxymethylcellulose, sorbitol and/or dextran, and, if necessary, also stabilisers.

The dose of the active ingredient depends on the warm-blooded animal species, the age and  
10 the individual condition and on the manner of administration. In the normal case, an approximate daily dose of about 10 mg to about 250 mg is to be estimated in the case of oral administration for a patient weighing approximately 75 kg .

The invention is described below, for the purpose of illustration only, in the following  
15 examples.

### Example 1

#### Determination of binding site preferences in zinc fingers

##### 20 *Design Of Zinc Finger Phage Display Libraries*

Zinc finger-DNA recognition at the interface between adjacent DNA subsites is studied using a zinc finger phage display library. This library is based on the three-finger DNA-binding domain of Zif268, but contains randomisations of amino acids from finger 2 (F2) and finger 3 (F3), at residue positions which could form a network of contacts across the  
25 interface of their DNA subsites. The detailed design of the library is shown in Figure 1c, together with the generic DNA binding site used in selections. Briefly, the library contains randomisations at F2 residue position 6 (hereafter denoted F2[+6]) and F3 residue positions -1, +1, +2 and +3 (hereafter denoted F3[-1], F3[+2], etc.).

30 Library selections are carried out using DNA binding sites that resembled the Zif268 operator, but which contained systematic combinations of bases in the DNA doublet which

forms the base-step between the DNA subsites of F2 and F3. DNA binding sites are of the generic form 5'-GNX-XCG-GCG-3', where X-X denotes a given combination of the bases at the interface between the DNA subsites, and N denotes that the four bases are equally represented at DNA position 3. Thus the interaction between F3[+3] and nucleotide position 3N is allowed complete freedom in this experiment. This feature of the library allows selection of a large family (or database) of related zinc fingers that bind a given combination of bases at nucleotide positions 4X and 5X, but which are non-identical owing to different interactions with the middle base in the nominal triplet subsite of F3.

- 10 The first library to be constructed, LIB-A, contains randomisations at F2 residue position 6 and F3 residue positions -1, 1, 2 and 3 (see Figure 2), and is sorted using the DNA sequence 5'GNX-XCG-GCG-3', where X-X denotes a known combination of the two bases at DNA positions 4X and 5X, and N denotes an equal probability of any of the four bases at DNA position 3. The second library, LIB-B, contains randomisations at F2 residue position 6 and F3 residue positions -1 and 2, and is sorted using the DNA sequence 5'-GCX-XCG-GCG-3', where X-X denotes a known combination of the two bases at DNA positions 4X and 5X.

The genes for the two different zinc finger phage display libraries are assembled from four synthetic DNA oligonucleotides by directional end-to-end ligation using three short complementary DNA linkers. The oligonucleotides contain selectively randomised codons (of sequence NNS; N = A/C/G/T, S = G/C) in the appropriate amino acid positions of fingers 2 and 3. The constructs are amplified by PCR using primers containing *Not I* and *Sfi I* restriction sites, digested with the above endonucleases to produce cloning overhangs, and ligated into phage vector Fd-Tet-SN. Electrocompetent *E. coli* TG 1 cells are transformed with the recombinant vector and plated onto TYE medium (1.5% agar, 1% Bacto tryptone, 0.5% Bacto yeast extract, 0.8% NaCl) containing 15 µg/ml tetracycline.

30 Allowing this freedom to some protein-DNA interactions that are not being studied is a useful strategy towards increasing the diversity of clones which can be obtained from any one selection experiment. However, at the same time, it is important to limit the number

of contacts that are allowed contextual freedom at any one time, otherwise there is a danger that a subset of particularly strong intermolecular interactions will dominate the selections. Anticipating this eventuality, a smaller sublibrary is also created that contains randomised residues only in positions F2[+6] and F3[-1 and +2], and therefore does not allow for contextual freedom in selections. Clones selected from this library are marked with an asterisk when they are discussed herein.

### *Experimental Strategy*

Phage selections from the two zinc finger libraries are performed separately in order to determine the diversity of DNA sequences which can be bound specifically by members of each library. Sixteen selections are performed on each library, using the different DNA binding sites that correspond to all 16 possible combinations of bases at nucleotide positions 4X and 5X. The DNA binding site used to select specifically binding phage is immobilised on a solid surface, while a 10-fold excess of each of the other 15 DNA sites is present in solution as a specific competitor.

### *Phage Selections*

Tetracycline resistant colonies are transferred from plates into 2xTY medium (16g/litre Bacto tryptone, 10g/litre Bacto yeast extract, 5g/litre NaCl) containing 50µM ZnCl<sub>2</sub> and 15 µg/ml tetracycline, and cultured overnight at 30°C in a shaking incubator. Cleared culture supernatant containing phage particles is obtained by centrifuging at 300g for 5 minutes.

Biotinylated DNA target sites (1pmol) are bound to streptavidin-coated tubes (Boehringer Mannheim). Phage supernatant solutions are diluted 1:10 in PBS selection buffer (PBS containing 50µM ZnCl<sub>2</sub>, 2% Marvel, 1% Tween, 20µg/ml sonicated salmon sperm DNA, 10 pmol/ml of each of the 15 other possible unbiotinylated DNA sites), and 1 ml is applied to each tube for 1 hour at 20°C. After this time, the tubes are emptied and washed 20 times with PBS containing 50µM ZnCl<sub>2</sub>, 2% Marvel and 1% Tween. Retained phage are eluted in 0.1ml 0.1M triethylamine and neutralised with an equal volume of 1M Tris (pH 7.4). Logarithmic-phase *E. coli* TG 1 (0.5ml) are infected with eluted phage (50µl), and used to prepare phage supernatants for subsequent rounds of selection. After 3 rounds of



selection, *E. coli* infected with selected phage are plated, individual colonies are picked and used to grow phage for binding site signature assays and DNA sequencing.

- After three rounds of phage selection against a particular DNA binding site, individual zinc finger clones are recovered, and the DNA binding specificity of each clone is determined by the binding site signature method. This involves screening each zinc finger phage for binding to eight different libraries of the DNA binding site, designed such that each library contains one fixed base and one randomised base at either of positions 4X and 5X (i.e. libraries GN, AN, TN, CN, and NG, NA, NT, NC). Thus each of the 16 DNA binding sites used in selection experiments is specified by a unique combination of two libraries - for example, the DNA binding site containing 4G5G is present in only two of the eight libraries in which the relevant doublet had one nucleotide randomised and the other nucleotide fixed as guanine, i.e. libraries 4G5N and 4N5G. The eight DNA libraries used in binding site signatures are arrayed across a microtitre plate and zinc finger phage binding is detected by phage ELISA. The pattern of binding to the eight DNA libraries reveals the DNA sequence specificity (or preference) of each phage clone, and only those clones found to be relatively specific are subsequently sequenced to reveal the identity of the amino acids present in the randomised zinc finger residue positions.
- Procedures are as described previously (Choo, Y. & Klug, A. (1994) Proc. Natl. Acad. Sci. USA 91, 11163-11167; Choo, Y. & Klug, A. (1994) Proc. Natl. Acad. Sci. USA 91, 11168-11172). Briefly, 5'-biotinylated positionally randomised oligonucleotide libraries, containing Zif268 operator variants, are synthesised by primer extension as described. DNA libraries (0.4 pmol/well for LIB-A and 1.2 pmol/well for LIB-B) are added to streptavidin-coated ELISA wells (Boehringer-Mannheim) in PBS containing 50µM ZnCl<sub>2</sub> (PBS/Zn). Phage solution (overnight bacterial culture supernatant diluted 1:10 in PBS/Zn containing 2% Marvel, 1% Tween and 20µg/ml sonicated salmon sperm DNA) are applied to each well (50µl/well). Binding is allowed to proceed for one hour at 20°C. Unbound phage are removed by washing 6 times with PBS/Zn containing 1% Tween, then 3 times with PBS/Zn. Bound phage are detected by ELISA using horseradish peroxidase-

conjugated anti-M13 IgG (Pharmacia Biotech) and the colourimetric signal quantitated using SOFFMAX 2.32 (Molecular Devices).

5 The coding sequence of individual zinc finger clones is amplified by PCR using external primers complementary to phage sequence. These PCR products are sequenced manually using Thermo Sequenase cycle sequencing (Amersham Life Science).

#### *Analysis Of Phage-Selected Zinc Fingers*

Figure 3 shows the binding site signatures of relatively sequence-specific zinc finger phages selected from both libraries, using the 16 different DNA doublets which form the base-step between the DNA subsites of fingers 2 and 3. The results show that zinc finger clones are selected which bind specifically to almost all subsites, including those triplets in which the 5' position (nucleotide 5X in the model system) is fixed as a base other than guanine. Overall, the selections show that any of the four bases can be bound specifically in both the 15 5' and 3' positions of a nominal triplet subsite. The results are summarised in Figure 4.

Selections from the smaller sub-library yield fingers that can bind specifically to only 8 of the 16 doublets, whereas members of the larger library yield fingers that recognise 15 out of the 16 doublets. It is not known whether this difference in efficacy originates from the inclusion of more randomised positions in the larger library, or the conformational flexibility afforded by the contextual freedom designed into the larger library, or both. The only base-step that does not yield specific zinc fingers is 4G5A. This dinucleotide may induce an unfavourable DNA deformation in the context of the DNA binding sites used for selection.

25

#### Example 2

##### Construction of a zinc finger protein

30 The target selected for the zinc finger nucleic acid binding protein is the activating point mutation of the human EJ bladder carcinoma *ras* oncogene, which was the first DNA lesion reported to confer transforming properties on a cellular proto-oncogene. Since the original

discovery, *ras* gene mutations have been found to occur at high frequencies in a variety of human cancers and are established targets for the diagnosis of oncogenesis at early stages of tumour growth.

- 5 The EJ bladder carcinoma mutation is a single nucleotide change in codon 12 of H-*ras*, which results in a mutation from GGC to GTC at this position. A zinc finger peptide is designed to bind a 9bp DNA site assigned in the noncoding strand of the mutant *ras* gene, such that three fingers contact 'anticodons' 10, 11 and 12 in series, as shown in Fig. 5. The rationale of this assignment takes into account the fact that zinc fingers make most contacts  
10 to one DNA strand, and the mutant noncoding strand carries an adenine which can be strongly discriminated from the cytosine present in the wild-type *ras*, by a bidentate contact from an asparagine residue.

- The first finger of the designer lead peptide is designed according to the rules set forth  
15 herein to bind the triplet 5'-GCC-3', which corresponds to 'anticodon' 10 of the designated binding site. The finger has the following sequence:

F Q C R I C M R N F S D R S S L T R H T R T H T G E K P  
-1 1 2 3 4 5 6 7 8 9

- 20 A DNA coding sequence encoding this polypeptide is constructed from synthesised oligonucleotides.

- Given the similarity of the DNA subsites, the second and third fingers of the DNA-binding  
25 domain are direct repeats of this first finger, but in which the third  $\alpha$ -helical residue which contacts the middle nucleotide of a triplet, +3, is mutated according to recognition rules, to histidine in finger 2 and asparagine in finger 3, such that the specificity of these fingers is predicted to be 5'-GGC-3' ('anticodon' 11) and 5'-GAC-3' ('anticodon' 12) respectively. Thus, the second and third finger polypeptides have the sequences

- 30 F Q C R I C M R N F S D R S H L T R H T R T H T G E K P

and

F Q C R I C M R N F S D R S N L T R H T R T H T G E K

5

respectively.

A construct consisting of DNA sequences encoding the three fingers joined together, preceded by a leader MAEEKP at the N-terminus, is cloned as a fusion to the minor coat protein (gene III) of bacteriophage Fd in the phage vector Fd-Tet-SN ( Y. Choo, A. Klug, (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11163-11167). In phage display screening, the DNA-binding domain is able to bind the mutated ras sequence with an apparent  $K_d$  of 17nM, and to discriminate strongly against the wild-type sequence.

15

### Example 3

#### Improvement of binding performance by selective randomisation

While a  $K_d$  of 17nM is sufficient for most practical applications of DNA-binding proteins, the apparent affinity of the designed protein falls about 5-fold short of the  $K_d$ s in the nanomolar range which are found for the reaction of wild-type zinc finger proteins with their natural binding sites ( Y. Choo, A. Klug, (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11168-11172).

25

According to the recognition rules, the first finger of the lead peptide could contact cytosine using one of Asp, Glu, Ser or Thr in the third  $\alpha$ -helix position. To determine the optimal contact, the codon for helical position 3 of finger 1 is engineered by cassette mutagenesis to have position 1 = A/G, position 2 = A/C/G and position 3 = C/G. Therefore in addition to Asp, Glu, Ser and Thr, the randomisation also specifies Ala, Arg, Asn, Gly and Lys. Selections from this mini-library are over one round of phage binding to 5nM mutant DNA oligo in 100  $\mu$ l PBS containing 50 $\mu$ M  $ZnCl_2$ , 2% (w/v) fat-free dried milk

30

(Marvel) and 1% (v/v) Tween-20, with 1µg poly dIdC as competitor, followed by six washes with PBS containing 50µM ZnCl<sub>2</sub> and 1% (v/v) Tween-20. Bound phage are eluted with 0.1M triethylamine for 3 mins, and immediately transferred to an equal volume of 1M Tris-Cl pH 7.4.

5

A single round of randomisation and selection is found to be sufficient to improve the affinity of the lead zinc finger peptide to this standard. A small library of mutants is constructed with limited variations specifically in the third α-helical position (+3) of finger 1 of the designed peptide. Selection from this library yields an optimised DNA-binding domain with asparagine at the variable position, which is able to bind the mutant *ras* sequence with an apparent K<sub>d</sub> of 3nM, i.e. equal to that of the wild-type Zif268 DNA-binding domain (Fig. 6). The selection of asparagine at this position to bind opposite a cytosine is an unexpected deviation from the recognition rules, which normally pair asparagine with adenine.

15

The selection of asparagine is, however, consistent with physical considerations of the protein-DNA interface. In addition to the classical bidentate interaction of asparagine and adenine observed in zinc finger-DNA complexes, asparagine has been observed to bridge a base-pair step in the major groove of DNA, for example in the co-crystal structures of the GCN4 DNA-binding domain. A number of different base-pair steps provide the correct stereochemical pairings of hydrogen bond donors and acceptors which could satisfy asparagine, including the underlined step GCC of *ras* 'anticodon' 10. Although asparagine in position 3 of the zinc finger helix would not normally be positioned to bridge a base-pair step according to the Zif268 model, it is known that a bend in DNA can give scope to non-canonical zinc finger-DNA interactions ( L. Fairall, J. W. R. Schwabe, L. Chapman, J. T. Finch, D. Rhodes, (1993) *Nature* 366, 483-487). The sequence GGC (codon 10) is frequently found on the outside of a bend in the nucleosome core, and has been observed to confer an intrinsic bend in the crystal structure of a decameric DNA oligonucleotide. In the latter case, the bend arises from preferential stacking of the purines: this is associated with a large propeller twist and narrowing of the major groove, both of which would favour bridging of the base-pair step by asparagine ( T. E. Ellenberger, C. J. Brandl, K. Struhl, S.

30

C. Harrison, (1992) *Cell* 71, 1223-1237). Therefore, in addition to explaining the selection of the non-canonical contact in the optimised complex, the sequence-dependent deformation of ras DNA could account for our observation that wild-type and EJ ras gene fragments have different electrophoretic mobility in polyacrylamide gels, since the wild-type ras gene has two GGC sequences 5 bp apart and hence out of helical phase (resulting in no net bend), while the EJ mutation affects one of these GGC triplets.

Thus, while it is possible to engineer an adequate DNA-binding domain by rational design based on recognition rules, the binding affinity of this lead peptide is improved using phage display leading to the selection of a non-canonical DNA contact.

#### Example 4

##### Diagnosis of a ras mutation using the zinc finger nucleic acid binding protein

- 15 The optimised DNA-binding domain displayed on phage is applied in the diagnosis of the activating point mutation of the EJ *ras* oncogene. Bacterial culture supernatant containing the diagnostic phage is diluted 1:1 with PBS containing 50 $\mu$ M ZnCl<sub>2</sub>, 4% (w/v) fat-free dried milk (Marvel) and 2% (v/v) Tween-20. Biotinylated oligonucleotides (7.5pmol) containing double stranded DNA comprising codons 8-16 from the wild type or the point-
- 20 mutated *ras* gene are added to 50 $\mu$ l of the diluted phage and incubated for 1h at 20°C. In the experiment shown in Fig. 3, bound phage are captured with 0.5mg streptavidin coated paramagnetic beads (Dyna) - however streptavidin coated microtitre plates (Boehringer Mannheim) can also be used without alteration to the protocol. Unbound phage are removed by washing the beads 6 times with PBS containing 50 $\mu$ M ZnCl<sub>2</sub> and 1% (v/v)
- 25 Tween-20. The beads are subsequently incubated for 1h at RT with anti-M13 IgG conjugated to horseradish peroxidase (Pharmacia Biotech) diluted 1:5000 in PBS containing 50 $\mu$ M ZnCl<sub>2</sub> and 2% (w/v) fat-free dried milk (Marvel). Excess antibody is removed by washing 6 times with PBS containing 50 $\mu$ M ZnCl<sub>2</sub> and 0.05% (v/v) Tween, and 3 times with PBS containing 50 $\mu$ M ZnCl<sub>2</sub>. The ELISA is developed with 0.1mg/ml
- 30 tetramethylbenzidine (Sigma) in 0.1M sodium acetate pH5.4 containing 2 $\mu$ l of fresh 30%

hydrogen peroxide per 10ml buffer, and after approximately 1 min, stopped with an equal volume of 2M H<sub>2</sub>SO<sub>4</sub>. The reaction produces a yellow colour which is quantitated by subtracting the absorbance at 650nm from the absorbance at 450nm. It should be noted that in this protocol the ELISA is not made competitive, however, soluble (non biotinylated) wild-type *ras* DNA could be included in the binding reactions, possibly leading to higher discrimination between wild-type and mutant *ras*.

Phage are retained specifically by DNA bearing the mutant, but not the wild-type *ras* sequence, allowing the detection of the point mutation by ELISA (Fig. 7).

#### Example 5

##### Design of an anti-HIV zinc finger

The sequence of the HIV TAR, the region of the LTR which is responsible for trans-activation by Tat, is known (Jones and Peterlin, (1994) Ann. Rev. Biochem. 63:717-743). A sequence with the TAT region is identified and a zinc finger polypeptide designed to bind thereto.

The selected sequence is 5' - AGA GAG CTC - 3', which is the complement of nucleotides +34 to +42 of HIV. The corresponding amino acids required in fingers 1, 2 and 3 of a zinc finger binding protein are determined according to the rules set forth above, as follows:

Finger 3:	target	5' - AGA - 3'	
25	Position -1	Gln	
	Position +2	Gly	
	Position +3	His	
	Position +6	Val	
30	Finger 2:	target	5' - GAG - 3'
	Position -1	Arg	

Position +2 Ser

Position +3 Asn

Position +6 Arg

5 Finger 1: target 5' - CTC - 3'

Position -1 Asp

Position +3 Ser

Position +6 Glu

10 The framework of the polypeptide is taken from the Zif 268 middle finger. The sequence of the entire polypeptide is shown in SEQ. ID No. 2.

Residues +2 and +6 of finger 3 are partially selected by randomisation and phage display selection. At position 2, two triplets are used, GAT and GGT, coding for Asp or Gly.

15 Position +6 was randomised. In these positions, the residues Gly and Val are selected. The methodology employed is as follows: colony PCR is performed with one primer containing a single mismatch to create the required randomisations in finger 3. Cloning of PCR product in phage vector is as described previously (Choo, Y. & Klug, A. (1994) Proc. Natl. Acad. Sci. USA 91, 11163-11167; Choo, Y. & Klug, A. (1994) Proc. Natl. Acad. Sci. USA 91, 11168-11172). Briefly, forward and backward PCR primers contained unique restriction sites for *Not* I or *Sfi* I respectively and amplified an approximately 300 base pair region encompassing three zinc fingers. PCR products are digested with *Sfi* I and *Not* I to create cohesive ends and are ligated to 100ng of similarly digested fd-Tet-SN vector. Electrocompetent TG1 cells are transformed with the recombinant vector. Single colonies of transformants are grown overnight in 2xTY containing 50µM ZnCl<sub>2</sub> 15µg/ml tetracycline. Single stranded DNA is prepared from phage in the culture supernatant and sequenced with Sequenase 2.0 (United States Biochemical).

25 The polypeptide designed according to the invention is then tested for binding to HIV DNA and positive results are obtained.



**Example 6****Design of a zinc finger specific for an 8bp palindrome**

Arrays of zinc fingers bind to asymmetric DNA sequences but are not known to bind  
5 palindromes. In order to determine whether an array of zinc fingers can bind to a  
palindrome a three finger domain is engineered to recognise the 8bp palindromic sequence  
GCGGCCGC which is bound and cleaved by the restriction endonuclease *NorI*.

A zinc finger domain is selected from the a Zif268 middle finger library (see WO  
10 96/06166) to bind the middle triplet GCC in the context of the Zif268 binding site. The  
sequence bound by this domain is GCG-GCC-GCG.

In order to do change the specificity of the zinc finger to the *NorI* recognition sequence  
GCG-GCC-GC the N-terminus of the  $\alpha$ -helix of finger 1 (F1) is mutated from position -2  
15 through to +2. Position -2 is Ser in WT Zif268 and could make a water mediated H-bond  
to a DNA phosphate: this is mutated to Arg in order to make a direct phosphate contact.  
Positions -1, 1 and 2 are mutated to Gly or Ala (Gly for -1 which is just outside the helix,  
and Ala for the other positions on the helix) in order to eliminate H-bonding groups which  
might function in DNA recognition. The protein is able to accept any base (N) in the  
20 sequence GCG-GCC-GCN with a small preference for A over G/T/C. The binding  
strength is not affected, even though the Arg->G contact of WT Zif268 is deleted, owing  
to compensation from the engineered phosphate contact. Thus a protein that bound the 8bp  
palindromic recognition site of *NotI* is engineered from a three finger domain based on  
Zif268.

25

The zinc finger domain selected from the library originally had Ser at position +3 of F2  
and recognises the sequence GCGGYC-GCG where Y is C or T. Since recognition of the  
*NorI* site requires specifying C at that DNA position, the mutation Ser->Asp is made at  
position +3 of F2 to narrow the DNA binding specificity from Y to C. This mutation is  
30 according to the rules set forth above. The final construct binds the sequence GCG-GCC-  
GC specifically.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

(A) NAME: MEDICAL RESEARCH COUNCIL

(B) STREET: 20 Park Crescent

(C) CITY: London

10

(E) COUNTRY: UK

(F) POSTAL CODE (ZIP): W1N 4AL

(G) TELEPHONE: +44 171 636 5422

(H) TELEFAX: +44 171 323 1331

15

(ii) TITLE OF INVENTION: Nucleic Acid Binding Proteins

(iii) NUMBER OF SEQUENCES: 2

## (iv) COMPUTER READABLE FORM:

20

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH: 264 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

35

(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5 (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..264

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCA GAA GAG AAG CCT TTT CAG TGT CGA ATC TGC ATG CGT AAC TTC AGC 48  
 Ala Glu Glu Lys Pro Phe Gln Cys Arg Ile Cys Met Arg Asn Phe Ser  
 1 5 10 15

15 GAT CGT AGT AGT CTT ACC CGC CAC ACG AGG ACC CAC ACA GGC GAG AAG 96  
 Asp Arg Ser Ser Leu Thr Arg His Thr Arg Thr His Thr Gly Glu Lys  
 20 25 30

20 CCT TTT CAG TGT CGA ATC TGC ATG CGT AAC TTC AGC AGG AGC GAT AAC 144  
 Pro Phe Gln Cys Arg Ile Cys Met Arg Asn Phe Ser Arg Ser Asp Asn  
 35 40 45

25 CTT ACG AGA CAC CTA ACG ACC CAC ACA GGC GAG AAG CCT TTT CAG TGT 192  
 Leu Thr Arg His Leu Arg Thr His Thr Gly Glu Lys Pro Phe Gln Cys  
 50 55 60

30 CGA ATC TGC ATG CGT AAC TTC AGG CAA GCT GAT CAT CTT CAA GAG CAC 240  
 Arg Ile Cys Met Arg Asn Phe Arg Gln Ala Asp His Leu Gln Glu His  
 65 70 75 80

CTA AAG ACC CAC ACA GGC GAG AAG 264  
 Leu Lys Thr His Thr Gly Glu Lys  
 85

35

42

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 88 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ala Glu Glu Lys Pro Phe Gln Cys Arg Ile Cys Met Arg Asn Phe Ser  
1 5 10 15

Asp Arg Ser Ser Leu Thr Arg His Thr Arg Thr His Thr Gly Glu Lys  
15 20 25 30

Pro Phe Gln Cys Arg Ile Cys Met Arg Asn Phe Ser Arg Ser Asp Asn  
35 40 45

Leu Thr Arg His Leu Arg Thr His Thr Gly Glu Lys Pro Phe Gln Cys  
50 55 60

Arg Ile Cys Met Arg Asn Phe Arg Gln Ala Asp His Leu Gln Glu His  
65 70 75 80

Leu Lys Thr His Thr Gly Glu Lys

## Claims:

1. A method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a nucleic acid base triplet in a target nucleic acid sequence,  
5 wherein binding to the 5' base of the triplet by an  $\alpha$ -helical zinc finger nucleic acid binding motif in the protein is determined as follows:
  - a) if the 5' base in the triplet is A, then position +6 in the  $\alpha$ -helix is Glu, Asn or Val;
  - b) if the 5' base in the triplet is C, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val, Ala,  
10 Glu or Asn.
2. A method according to claim 1, wherein binding to the 5' base of the triplet by an  $\alpha$ -helical zinc finger nucleic acid binding motif in the protein is additionally determined as follows:  
15
  - a) if the 5' base in the triplet is G, then position +6 in the  $\alpha$ -helix is Arg or Lys;
  - b) if the 5' base in the triplet is T, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val or Lys.
3. A method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger  
20 class capable of binding to a nucleic acid triplet in a target nucleic acid sequence, wherein binding to each base of the triplet by an  $\alpha$ -helical zinc finger nucleic acid binding motif in the protein is determined as follows:
  - 25 a) if the 5' base in the triplet is G, then position +6 in the  $\alpha$ -helix is Arg or Lys;
  - b) if the 5' base in the triplet is A, then position +6 in the  $\alpha$ -helix is Glu, Asn or Val;
  - c) if the 5' base in the triplet is T, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val or Lys;
  - d) if the 5' base in the triplet is C, then position +6 in the  $\alpha$ -helix is Ser, Thr, Val, Ala,  
Glu or Asn;
  - 30 e) if the central base in the triplet is G, then position +3 in the  $\alpha$ -helix is His;
  - f) if the central base in the triplet is A, then position +3 in the  $\alpha$ -helix is Asn;

- g) if the central base in the triplet is T, then position +3 in the  $\alpha$ -helix is Ala, Ser or Val; provided that if it is Ala, then one of the residues at -1 or +6 is a small residue;
- h) if the central base in the triplet is C, then position +3 in the  $\alpha$ -helix is Ser, Asp, Glu, Leu, Thr or Val;
- 5 i) if the 3' base in the triplet is G, then position -1 in the  $\alpha$ -helix is Arg;
- j) if the 3' base in the triplet is A, then position -1 in the  $\alpha$ -helix is Gln;
- k) if the 3' base in the triplet is T, then position -1 in the  $\alpha$ -helix is His or Thr;
- l) if the 3' base in the triplet is C, then position -1 in the  $\alpha$ -helix is Asp or His.
- 10 4. A method according to any preceding claim, wherein the or each zinc finger has the general primary structure
- (A)  $X^a$  C  $X_{2-4}$  C  $X_{2-3}$  F  $X^c$  X X X X L X X H X X  $X^b$  H - linker
- 1 1 2 3 4 5 6 7 8 9
- 15 wherein X (including  $X^a$ ,  $X^b$  and  $X^c$ ) is any amino acid.
5. A method according to claim 5 wherein  $X^a$  is  $F/Y-X$  or  $P-F/Y-X$ .
- 20 6. A method according to claim 4 or claim 5 wherein  $X_{2-4}$  is selected from any one of: S-X, E-X, K-X, T-X, P-X and R-X.
7. A method according to any one of claims 4 to 6 wherein  $X^b$  is T or I.
- 25 8. A method according to any one of claims 4 to 7 wherein  $X_{2-3}$  is G-K-A, G-K-C, G-K-S, G-K-G, M-R-N or M-R.
9. A method according to any one of claims 4 to 8 wherein the linker is T-G-E-K or T-G-E-K-P.
- 30 10. A method according to any one of claims 4 to 9 wherein position +9 is R or K.

11. A method according to any one of claims 4 to 10 wherein positions +1, +5 and +8 are not occupied by any one of the hydrophobic amino acids, F, W or Y.
- 5 12. A method according to claim 11 wherein positions +1 +5 and +8 are occupied by the residues K, T and Q respectively.
13. A method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a target nucleic acid sequence, comprising the steps of:
- 10 a) selecting a model zinc finger domain from the group consisting of naturally occurring zinc fingers and consensus zinc fingers; and
- b) mutating the finger according to the rules set in any one of claims 1 to 3.
- 15 14. A method according to claim 13, wherein the model zinc finger is a consensus zinc finger whose structure is selected from the group consisting of the consensus structure P Y K C P E C G K S F S Q K S D L V K H Q R T H T G, and the consensus structure P Y K C S E C G K A F S Q K S N L T R H Q R I H T G E K P.
- 20 15. A method according to claim 13 wherein the model zinc finger is a naturally occurring zinc finger whose structure is selected from one finger of a protein selected from the group consisting of Zif 268 (Elrod-Erickson *et al.*, (1996) Structure 4:1171-1180), GLI (Pavletich and Pabo, (1993) Science 261:1701-1707), Tramtrack (Fairall *et al.*, (1993) Nature 366:483-487) and YY1 (Houbaviy *et al.*, (1996) PNAS (USA) 93:13577-13582).
- 25 16. A method according to claim 15 wherein the model zinc finger is finger 2 of Zif 268.
- 30 17. A method according to any preceding claim wherein the binding protein comprises two or more zinc finger binding motifs, placed N-terminus to C-terminus.

18. A method according to claim 14, wherein the N-terminal zinc finger is preceded by a leader peptide having the sequence MAEEKP.

5 19. A method according to claim 14 or claim 15, wherein the nucleic acid binding protein is constructed by recombinant DNA technology, the method comprising the steps of:

- 10 a) preparing a nucleic acid coding sequence encoding two or more zinc finger binding motifs as defined in any one of claims 5 to 13, placed N-terminus to C-terminus;
- b) inserting the nucleic acid sequence into a suitable expression vector; and
- c) expressing the nucleic acid sequence in a host organism in order to obtain the nucleic acid binding protein.

15 20. A method according to any preceding claim comprising the additional steps of subjecting the nucleic acid binding protein to one or more rounds of randomisation and selection in order to improve the characteristics thereof.

20 21. A method according to claim 20, wherein the randomisation and selection is carried out by phage display technology.

22. A method according to claim 21, comprising the steps of:

- 25 a) preparing a nucleic acid construct capable of expressing a fusion protein comprising the nucleic acid binding protein and a minor coat protein of a filamentous bacteriophage;
- b) preparing further nucleic acid constructs capable of expressing a fusion protein comprising a selectively mutated nucleic acid binding protein and a minor coat protein of a filamentous bacteriophage;
- 30 c) causing the fusion proteins defined in steps (a) and (b) to be expressed on the surface of bacteriophage transformed with the nucleic acid constructs;



d) assaying the ability of the bacteriophage to bind the target nucleic acid sequence and selecting the bacteriophage demonstrating superior binding characteristics.

23. A method according to any one of claims 20 to 22 wherein the nucleic acid binding  
5 protein is selectively randomised at any one of positions +1, +5, +8, -1, +2, +3 or +6.

24. A method according to claim 23, wherein, in the nucleic acid binding protein,  
position +6 of a zinc finger and positions -1, +1, +2 and +3 of an adjacent zinc finger  
are randomised.

10

25. A method for determining the presence of a target nucleic acid molecule,  
comprising the steps of:

a) preparing a nucleic acid binding protein by the method of any preceding claim which is  
15 specific for the target nucleic acid molecule;

b) exposing a test system comprising the target nucleic acid molecule to the nucleic acid  
binding protein under conditions which promote binding, and removing any nucleic acid  
binding protein which remains unbound;

c) detecting the presence of the nucleic acid binding protein in the test system.

20

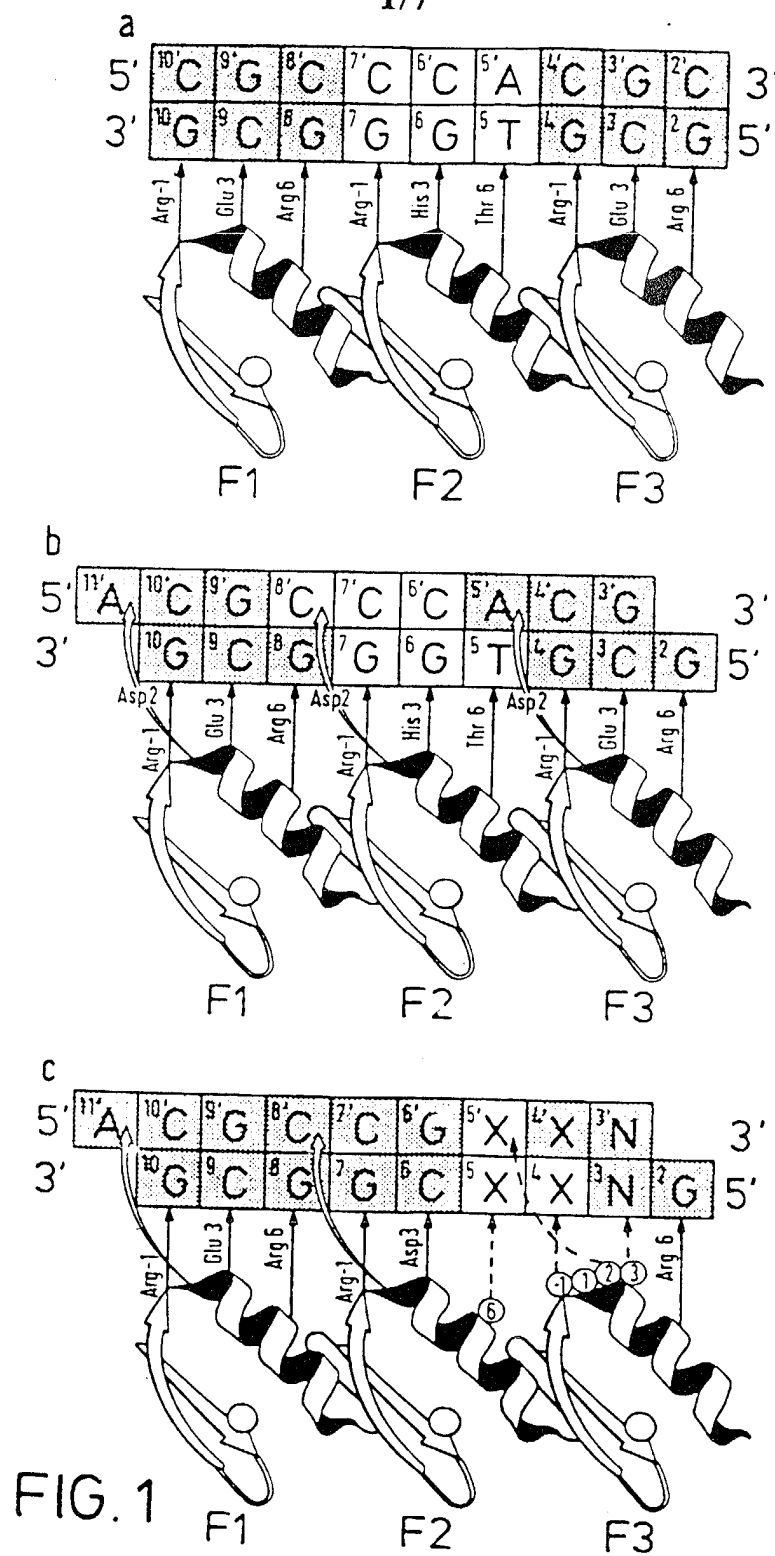
26. A method according to claim 25, wherein the presence of the nucleic acid binding  
protein in the test system is detected by means of an antibody.

27. A method according to claim 25 or claim 26 wherein the nucleic acid binding  
25 protein, in use, is displayed on the surface of a filamentous bacteriophage and the presence  
of the nucleic acid binding protein is detected by detecting the bacteriophage or a  
component thereof.

28. A synthetic nucleic acid binding protein whose design incorporates a method  
30 according to any one of claims 1 to 24.

29. A nucleic acid encoding a nucleic acid binding protein according to claim 28.
30. A host cell transformed with a nucleic acid according to claim 29.
- 5 31. Use of a nucleic acid binding protein according to claim 28 or a nucleic acid according to claim 29 in medicine.

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BASE AT POSITIONS 4'X AND 5'X OF DNA SITES USED IN SELECTIONS	AMINO ACIDS SELECTED IN FINGERS 2 AND 3	BINDING SITE SIGNATURE	No. OF CLONES SELECTED AND SCREENED	BASES AT POSITIONS 4'X AND 5'X OF DNA SITES USED IN SELECTIONS	AMINO ACIDS SELECTED IN FINGERS 2 AND 3	BINDING SITE SIGNATURE	No. OF CLONES SELECTED AND SCREENED
4'X 5'X	F2 F3	4'X 5'X		4'X 5'X	F2 F3	4'X 5'X	
GG	6 -1123 R RLEY * R RSED R RHTH R RSSE R RSSA	G A T C G A T C	4 4 1 1 1	CC	6 -1123 T DPHN E HSKS E HROH S DRAN T DRAN	G A T C G A T C	1 4 1 1 2
AG	R QVTT * R QSGD R QLAT R QDAH R QRAS K QSTS	A G	4 5 2 1 1 1	AC	* Y RSQD V QVGH T QLAT	A A	3 2 3
TG	R SSGD R SASA * R NSGD R LVON K TGAS R TPSG R TOTA R TSAA	T G	1 1 1 1 1 1 1 2	TA	N TSQA N TSAS K LAQT * E RSRD E RLRD V HLAT V HLTT L VGHH	T A	4 2 1 3 4 1 1 1
CG	R DTSV R DAST R DASA R DTSS	C G	3 2 1 2	CA	Y HPAT N HPAN E HMSN L DSRA	C A	2 1 9 2
GC	A RNHO * N RSTD * S RSTD S SRHS T RNST T RTST T TRYX T RAQN	G C	2 3 6 1 1 1 3 1	GT	* T KSSD * K SSSD * K RSHD A RSSY T RSSS T RKTD	G T	2 1 1 1 1 1
AC	A QAA A QGTN * V TGRD S ORGA S QSTT A TSSS T SSST	A C	4 1 4 1 2 1 1	AT	S QIST S QIGA T QYST T QSAS T QSQH T QTSN T QPGH T QDTH K QDST	A T	3 1 1 1 1 1 1 2
TC	D TISN N TSTA V TSSL V TSSI V TSNS * R GSND A TTSS T TAGS S TTSS S TSSA S LSTT G LSST	T C	1 1 4 1 3 1 1 1 1 1 1 1	TT	T TAST T TASH T TSSV T TSSA S HHTS S HAQT S HATT	T T	1 1 1 1 1 1 4
				CT	K DHSS T HPST S DSSR	C T	5 1 1

RELATIVE SIGNATURE STRENGTH ■ ≤1.00 ■ 0.80 ■ 0.60 ■ 0.40 □ 0.20 □ 0.00

FIG. 3

SUBSTITUTE SHEET (RULE 26)

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**a**

AMINO ACID SELECTED AT POSITION -1

BASE PRESENT AT POSITION <sup>4</sup> X IN BINDING SIGNATURES	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
G	0	0	0	0	0	0	0	0	1	0	0	0	0	0	18	2	2	0	0	0
A	0	0	0	0	0	0	0	0	0	0	0	0	0	21	1	1	1	0	0	0
T	0	0	0	0	0	1	7	0	0	4	0	1	0	2	5	3	21	1	0	0
C	0	0	10	0	0	0	7	0	0	2	0	0	0	0	2	0	0	0	0	0

↓                      ↓                      ↓                      ↓                      ↓

C                      T/C                      T                      A G(T)                      T

RECOGNITION PATTERNS

**b**

AMINO ACID SELECTED AT POSITION 6

BASE PRESENT AT POSITION <sup>5</sup> X IN BINDING SIGNATURES	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
G	0	0	0	0	0	0	0	0	4	1	0	0	0	0	21	0	0	0	0	0
A	0	0	0	3	0	0	0	0	1	2	0	3	0	0	0	0	2	3	0	2
T	1	0	0	0	0	1	0	0	5	0	0	0	0	0	0	10	17	3	0	0
C	5	0	1	3	0	1	0	0	0	0	0	3	0	0	2	12	14	6	0	0

↓                      ↓                      ↓                      ↓                      ↓                      ↓

C                      A/C                      G/T                      A/C                      G                      T/C                      C(A/T)

RECOGNITION PATTERNS

FIG. 4

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FIG. 5A

	8	9	10	11	12	13	14	15	16
Wild-type	5' GTG GTG GGC GCC GGC GGC	GGT GTG GGC AAG 3'							
	3' CAC CAC CCG CCG CCG CCG	CCA CAC CCG TTG 5'							

EJ mutant	5' GTG GTG GGC GCC GCC GTC GGT GTG GGC AAG 3'
	3' CAC CAC CCG CCG CCG CAG CCA CCA CCG TTG 5'



F1	M A E E K P	F O C R I C M R N F S D R S S L T R H T R T T	T H	T G E K P
F2		F O C R I C M R N F S D R S S L T R H T R T T	T H	T G E K P
F3		F O C R I C M R N F S D R S S L T R H T R T T	T H	T G E K

FIG. 5C

$\alpha$  - helix

$\beta$

$\beta$

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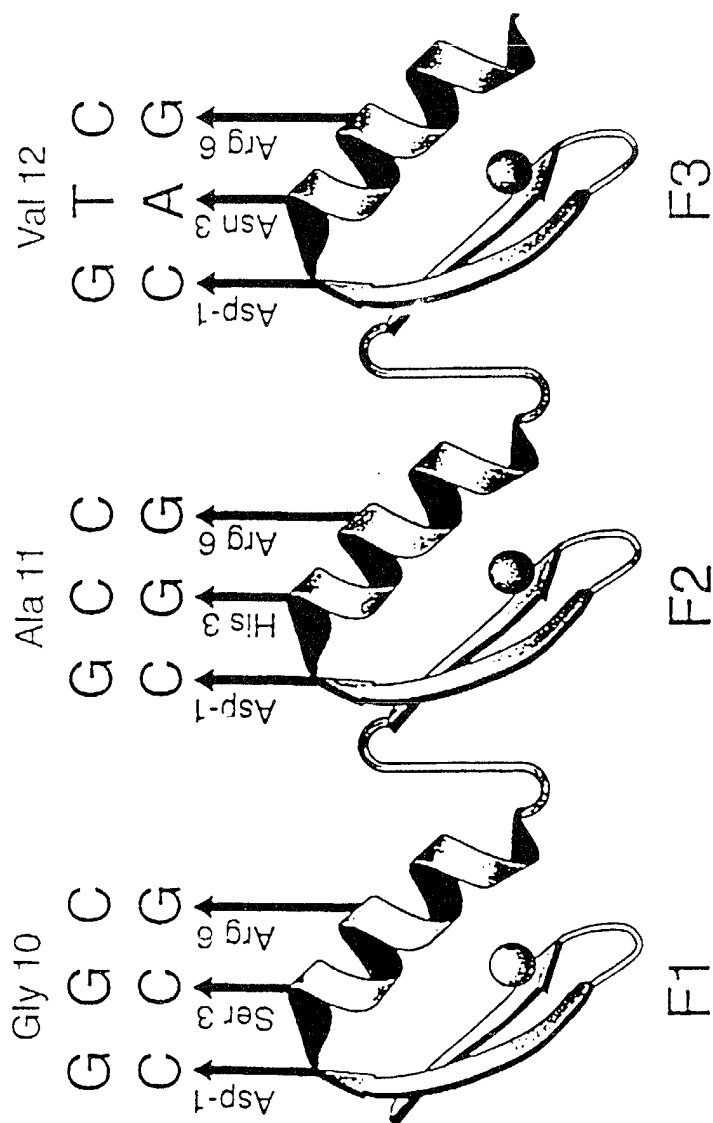


FIG. 5B



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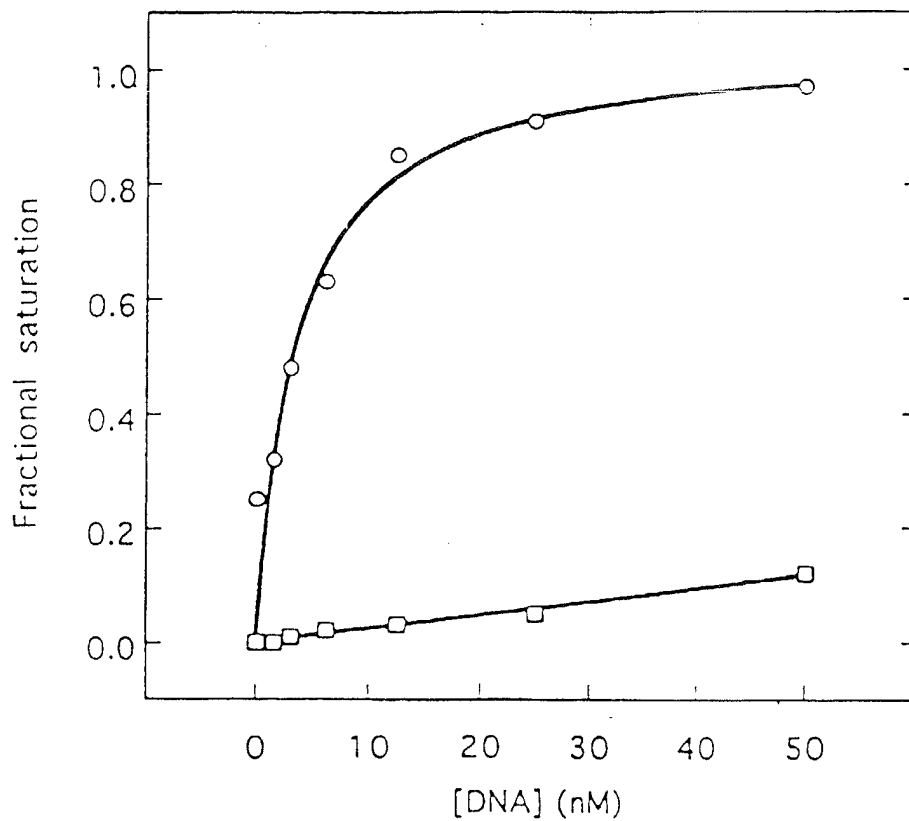


FIG. 6

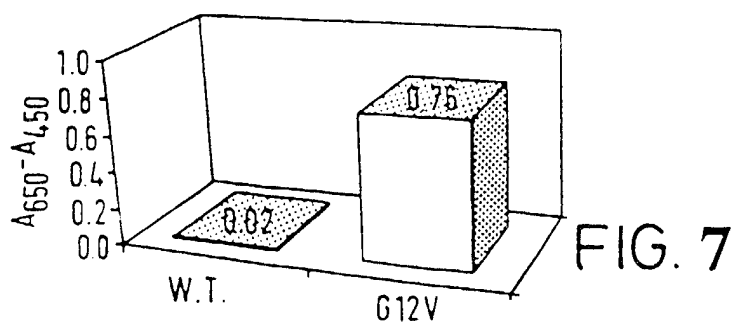


FIG. 7

# INTERNATIONAL SEARCH REPORT

Intern. Application No  
PCT/GB 98/01514

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/10 C12N15/12 C12N15/62 C12Q1/68 C07K14/47  
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
P, X	M. ISALAN ET AL: "Synergy between adjacent zinc fingers in sequence-specific DNA recognition" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 94, 27 May 1997, pages 5617-5621, XP002075337 WASHINGTON US see the whole document --- -/--	1-31

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex

### \* Special categories of cited documents

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "d" document member of the same patent family

Date of the actual completion of the international search

1 September 1998

Date of mailing of the international search report

30/09/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Fax: (+31-70) 340-3016

Authorized officer

Cervigni, S

# INTERNATIONAL SEARCH REPORT

Intern    al Application No  
PCT/GB 98/01514

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 96 06166 A (MEDICAL RES COUNCIL ;CHOO YEN (SG); KLUG AARON (GB); GARCIA ISIDRO) 29 February 1996 cited in the application see the whole document see page 5-6 see page 9, paragraph 3 see page 15, paragraph 2 see figures 2,7,15; tables 1,2</p> <p>---</p>	1-31
Y	<p>Y. CHOO, A. KLUG: "Physical basis of a protein-DNA recognition code" CURR. OP. STRUCT. BIOL., vol. 7, no. 1, February 1997, pages 117-125, XP002075338 see the whole document see page 122, column 1</p> <p>---</p>	1-31
Y	<p>CHOO Y ET AL: "Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 91 (23). 1994. 11168-11172. ISSN: 0027-8424, 8 November 1994, XP002075339 see figure 1</p> <p>---</p>	1-31
Y	<p>CHOO Y ET AL: "Toward a code for the interactions of zinc fingers with DNA: Selection of randomized fingers displayed on phage." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 91 (23). 1994. 11163-11167. ISSN: 0027-8424, 8 November 1994, XP002075340 see the whole document see figures 1,2 see page 11166</p> <p>---</p>	1-31
Y	<p>M. SUZUKI AND N. YAGI: "DNA recognition code of transcription factors in the helix-turn-helix ... and zinc finger families" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, 1994, pages 12357-12361, XP002075341 WASHINGTON US see the whole document see figure 1G</p> <p>---</p>	1-31

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# INTERNATIONAL SEARCH REPORT

Intern al Application No  
PCT/GB 98/01514

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	H. WU ET AL.: "Building zinc fingers by selection: Toward a therapeutic application" PROC. NATL.ACAD SCI., vol. 92, no. 2, 17 January 1995, pages 344-348, XP002075342 NATL. ACAD SCI., WASHINGTON, DC, US; see table 1 ---	
A	A.C. JAMIESON ET AL.: "In vitro selection of zinc fingers with altered DNA-binding specificity" BIOCHEMISTRY, vol. 33, no. 19, 17 May 1994, pages 5689-5695, XP002075344 AM. CHEM. SOC., WASHINGTON, DC, US see figure 2; table 2 ---	
A	IKEDA M ET AL: "A fusion protein library: an improved method for rapid screening and characterization of DNA binding or interacting proteins" GENE, vol. 181, no. 1-2, 28 November 1996, page 167-171 XP004071876 see abstract ---	
A	L. FAIRALL ET AL.: "The crystal structure of a two zinc-finger peptide reveals an extension to the rules for zinc-finger/DNA recognition" NATURE, vol. 366, 2 December 1993, pages 483-487, XP002075345 LONDON GB cited in the application see the whole document ---	
A	N.P.PAVLETICH ET AL: "Crystal structure of a five-finger GLI-DNA complex: new perspectives on Zinc fingers" SCIENCE., vol. 261, September 1993, pages 1701-1707, XP002075346 US cited in the application see the whole document ---	
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# INTERNATIONAL SEARCH REPORT

Intern al Application No  
PCT/GB 98/01514

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. ELROD-ERICKSON ET AL: "Zif268 protein-DNA complex refined at 1.6A: a model system for understanding zinc finger-DNA interactions" STRUCTURE, vol. 4, no. 10, 1996, pages 1171-1180, XP002075347 cited in the application see the whole document	
A	H.B. HOUBAVIY ET AL: "Cocrystal structure of YY1 bound to the adeno-associated virus P5 initiator" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, November 1996, pages 13577-13582, XP002075348 WASHINGTON US cited in the application see the whole document	

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 98/01514

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 31  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 31  
is directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/GB 98/01514

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9606166 A	29-02-1996	AU 3229195 A	14-03-1996
		CA 2196419 A	29-02-1996
		EP 0781331 A	02-07-1997
		JP 10504461 T	06-05-1998